COMPARISON OF CECAL COLONIZATION OF *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM IN WHITE LEGHORN CHICKS AND *SALMONELLA*-RESISTANT MICE

A Thesis

by

CHRISTINE PATRICIA SIVULA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2008

Major Subject: Laboratory Animal Medicine

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Approved by:

Co-Chairs of Committee,	Helene Andrews-Polymenis
	L. Garry Adams
Committee Member,	Melanie Ihrig
Head of Department,	Gerald R. Bratton

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ABSTRACT

Comparison of Cecal Colonization of *Salmonella enterica* serotype Typhimurium in White Leghorn Chicks and *Salmonella*-resistant Mice. (August 2008)

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Salmonellosis is one of the most important bacterial food borne illnesses worldwide. Among the many *Salmonella* serotypes, Typhimurium is the most commonly implicated serotype in human disease in the United States. A major source of infection for humans is consumption of chicken or egg products that have been contaminated with *S.* Typhimurium. The breadth of knowledge regarding colonization and persistence factors in the chicken is small when compared to our knowledge of factors that are important for these processes in other species used in *Salmonella* research, such as cattle and mice. Defining the factors important for these processes in the chick is the first step in decreasing the transmission of *Salmonella* between animal and human hosts.

In this work, we developed a chicken model to identify and study intestinal colonization and persistence factors of *Salmonella enterica* serovar Typhimurium. We studied the degree of enteric and systemic colonization of wild type *S.* Typhimurium ATCC14028, one of the most widely studied Typhimurium isolates, in White Leghorn

chicks and in *Salmonella*-resistant CBA/J mice during infection. Furthermore, we determined the distribution of wild type *S.* Typhimurium and a SPI-1 mutant (*invA*) during competitive infection in the cecum of 1-week-old chicks and 8-week-old mice. Cell associated, intracellular and luminal distributions of these strains in the cecum were analyzed as total counts in each compartment and also as a competitive index. Localization of *S.* Typhimurium ATCC14028 and the role of SPI-1 in colonization are well studied in murine models of infection, but comparative infection in chicks with the same strain has not been undertaken previously.

We show that the cecal contents are the major site for recovery of *S.* Typhimurium in the cecum of 1-week-old chicks and *Salmonella*-resistant mice. We also show that while SPI-1 is important for successful infection in the murine model, it is important only for cell association in the cecum of 1-week-old chicks. Finally, we found that in chicks infected at 1 week of age, bacterial counts in the feces do not reflect those seen in the cecum as they do in mice.

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Lydia Bogomolnaya, Megan Reynolds, and Hee-Jeong Yang deserve a special thank you for their guidance and assistance in the laboratory. I am grateful for your help on all aspects of this project.

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Finally, I would like to thank my family for their love and support. Thanks especially to my husband for moving all the way across the country to allow me to achieve this goal.

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1. INTRODUCTION

1.1 BACKGROUND

Salmonella species are gram-negative, motile, intracellular bacteria of the family enterobacteriaceae. There are two species in the genus *Salmonella*, *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies: *S. enterica* subspecies *enterica* (I), *S. enterica* subspecies *salamae* (II), *S. enterica* subspecies *arizonae* (IIIa), *S. enterica* subspecies *diarizonae* (IIIb), *S. enterica* subspecies *houtenae* (IV), and *S. enterica* subspecies *indica* (VI). There are currently 2,541 serovars of *Salmonella*,[26] over half of which belong to *S. enterica* subspecies *enterica*. Approximately 99% of all human and animal infections are caused by serovars in subspecies I.[6] While systemic illness in humans is caused by host adapted serovars *S.* Typhi and *S.* Paratyphi, many nontyphoidal serovars such as *S.* Typhimurium cause enteric disease in a wide rage of species including humans, cattle, swine and poultry.[24] The ability of *S.* Typhimurium and other nontyphoidal species to cause enteric disease lies in it's ability to invade the intestinal epithelial cells of the host.[13] Interaction between *Salmonella* and the host epithelium leads to a neutrophilic inflammatory response.[16]

There are an estimated 1.4 million cases annually of food related illness caused by *Salmonella* in the United States.[22] Salmonellosis generally causes mild to severe clinical disease in humans that is characterized by diarrhea, however infections can be fatal especially in immunocompromised persons. *S.* Typhimurium is among the most prevalent serotypes associated with food related illness along with *S.* Enteritidis and *S.*

This thesis follows the style of Comparative Medicine.

Heidelberg.[2,29] Consumption of contaminated food including poultry and egg products are often associated with clinical illness in humans.[29]

The mortality rate in newly hatched chickens infected with *S.* Typhimurium is high due to severe systemic disease with chemokine and proinflammatory cytokine expression in the gastrointestinal tract, liver and spleen.[40] However, in chicks over a few days of age, *S.* Typhimurium colonizes the gastrointestinal tract but does not cause clinical disease.[41] In this case, chicks are able to shed *Salmonella* into the environment for extended periods of time increasing the risk for contamination and dissemination into the food chain.

1.2 PATHOGENESIS OF ENTERIC SALMONELLOSIS

Following oral ingestion of *S.* Typhimurium, these bacteria adhere to and invade the intestinal epithelial cells and cells of intestinal lymphoid follicles or Peyer's patches via bacterial-mediated endocytosis.[12] Injection of effector proteins of the *Salmonella* Pathogenicity Island-1 (SPI-1) encoded type III secretion system (TTSS) into intestinal epithelial cells leads to cystoskeletal rearrangement in the host cell that result in the formation of membrane ruffles that engulf the bacteria and disrupt the brush border. Several genes encoding effector proteins, including SipB, SipC and SipD, have been identified that are required for invasion of enterocytes and are encoded within the SPI-1 type III secretion system.[20] In mice and other mammals, *Salmonella* enter both nonphagocytic enterocytes and microfold cells (M cells) associated with the Peyer's patches.[8] Interaction between *Salmonella* and host epithelial cells leads to the recruitment of macrophages and polymorphonuclear leukocytes (PMN) to the infected

intestine. Additionally, serotypes that cause enteritis induce fluid secretion into the intestinal lumen leading to an inflammatory diarrhea that is mediated by the SPI-1 TTSS.[1,32,33,36,37] In a calf model of *S.* Typhimurium enteritis, the SPI-1 TTSS effector proteins SipA, SopD, SopA, SopB, SopE and SopE2 play important roles in the induction of inflammation and fluid accumulation in the intestinal tract.[42] Serotypes that cause systemic infection enter macrophages where *Salmonella* Pathogenicity Island-2 (SPI-2) TTSS mediated replication and dissemination occur.[17,30]

1.3 ANIMAL MODELS

Animal models are a valuable tool used to study the pathogenesis of *S.* Typhimurium infection. When infected with *S.* Typhimurium, susceptible mice, including BALB/c and C57B/6 strains, develop a fatal systemic disease similar to that of Typhoid fever. Susceptible mice carry a non-resistant allele of the natural resistance associated macrophage protein (*NRAMP*) gene and thus are susceptible to systemic infection.[21] *Salmonella*-resistant mice, including strains CBA, A/J, and 129/sV, carry a wild type *NRAMP* allele and are resistant to development of disease following infection with *Salmonella*. Resistant strains are useful for long term studies involving persistence because the gastrointestinal tract is colonized with high numbers of organisms that are shed in the feces of infected mice.

Several species of livestock that are natural hosts of *Salmonella* are also used as models of *S.* Typhimurium infection. The calf is a useful model for studying *Salmonella* induced gastroenteritis because they develop an enteric disease similar to that of humans upon infection with *S.* Typhimurium.[28] Much information regarding pathophysiology of

Salmonella induced inflammatory gastrointestinal disease has been gained via this model.

Poultry generally become asymptomatically colonized with *S.* Typhimurium and are another useful model to understand the bacterial factors that promote commensalism in this species. Factors involved in intestinal colonization in mammalian species may not be important in avian species and vice versa, as infection with *S.* Typhimurium has different outcomes in thesis models. Research utilizing the chick model has included pathophysiology of infection in poultry as well as identification of colonization factors.[23,35] The inclusion of chickens as animal models is an important process in the effort to control transmission of *S.* Typhimurium in the poultry industry and subsequent contamination of the food supply.

1.4 SALMONELLOSIS IN THE CHICK

Whether or not a chick develops illness upon exposure to *Salmonella* depends on the infecting serovar, the age, the immune status and the genetic background of the chick. Host specific *S. enterica* serovar Gallinarum and serovar Pullorum cause fowl typhoid in the adult bird and pullorum disease in the newly hatched chick, respectively. Other strains including broad host range *S. enterica* serovar Typhimurium may also cause illness in chickens. In newly hatched chicks, *S.* Typhimurium replicate in the intestinal tract and *Salmonella* invade the intestinal mucosa.[4] Invasion leads to systemic disease characterized by CXC cytokine expression and a PMN influx.[40] Death in young chicks is attributed to anorexia and dehydration. After 3 days of age, chicks are relatively resistant to infection with *S.* Typhimurium which is presumed to be due to

maturation of macrophages and the development of commensal flora in the gut leading to competitive exclusion.[3] In older chicks, *Salmonella* colonize the gastrointestinal tract leading to contamination of the environment but do not cause clinical disease.

In chickens, the cecum is one of the major sites of *Salmonella* colonization. Following oral inoculation, *S. enterica* serovar Enteritidis and serovar Thompson pass across the cecal mucosa in newly hatched chicks.[25] *S.* Typhimurium is capable of invading the cecal mucosa and can be found in the epithelium, sub-epithelial and lamina propria basement membrane regions of the cecum.[5] Recovery of organisms following oral inoculation of chicks with *Salmonella* is also greatest in the cecum.[4,7,10,11,34] This localization is likely because the cecum is one of the main organs for epithelial adherence of organisms following inoculation.[31]

1.5 COLONIZATION FACTORS IN THE CHICK

Certain colonization factors that have been found to be important for intestinal invasion in mammals may not be important for colonization of the chick. The SPI-1 TTSS encodes the structural genes for the type III secretion system encoded by the *inv* and *spa* loci. This specialized protein machinery is responsible for the secretion of effector proteins, encoded on SPI-1 and elsewhere on the bacterial chromosome, necessary for invasion of the intestinal epithelium by *Salmonella*. The role of SPI-1 TTSS in serotype Typhimurium is well described in mammals. *In vitro* studies as well as *in vivo* mouse and bovine models of infection have shown that a functional SPI-1 TTSS in Typhimurium is necessary for epithelial cell invasion and induction of cytokines.[13,14,15,37,38]

However, the role of SPI-1 TTSS in Typhimurium infection is less clear in chickens and it is possible that factors other than SPI-1 are more important for commensal intestinal colonization in this species. Mutants in *invA*, *invB* and *invC* genes, encoding structural components of the SPI-1 type III secretion system, had reduced oral virulence and colonization by *S.* Enteriditis and *S.* Typhimurium (UK-1) in 1-day-old specific-pathogen-free (SPF) white leghorn chicks while mutations of the structural gene *invH* had no effect on virulence or colonization.[27] Mutants in the SPI-1 structural gene *spaS* were shown to be involved in systemic infection and intestinal colonization of *S.* Typhimurium (F98) in 1-day-old and 1-week-old chicks respectively but were not essential for either process.[18] Although the mutant had little effect in 1-day old chicks, it showed reduced levels of systemic and gastrointestinal colonization in 1-week old birds. Typhimurium (F98) mutants in *sipC*, a SPI-1 TTSS effector gene, were defective for colonization in 3-week-old SPF Light Sussex chickens.[35]

In contrast, the *spaS* gene was not required for *S.* Gallinarum to cause systemic disease in three-week-old SPF Rhode Island Red chickens or for virulence of *S.* Pullorum in 1-day-old commercial Brown egg layers.[19,39] Very few SPI-1 TTSS encoded genes were found to be necessary for colonization of 2-week-old SPF Light Sussex chicks by *S.* Typhimurium in a signature-tagged mutagenesis screen while many of these genes were required for colonization of cattle.[23] Several additional colonization factors have been found to be important in the chicken including many genes involved in lipopolysaccharide synthesis as well as fimbrial, metabolic and regulatory genes.[23,35]

1.6 OBJECTIVES

The objective of this study was to develop a 1-week-old SPF White Leghorn chick model of infection with ATCC14028 *Salmonella enterica* serotype Typhimurium. We used this model to characterize systemic and intestinal colonization by ATCC14028 in the chick during infection and to compare this to colonization of a well studied murine model. In addition, we used this model to determine the distribution of SPI-1 mutants with a nonfunctional TTSS-1 in a direct comparison in chicks and mice. Finally, we used this model to compare the levels of fecal shedding and cecal colonization in both models.

2. MATERIALS AND METHODS

2.1 ANIMALS

The procedures described in this study were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC). Animals were housed in an IACUC approved facility that is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

One-week-old unsexed specific pathogen free White Leghorn chicks were obtained from Charles River SPAFAS (North Franklin, CT). They were housed in a poultry brooder (Alternative Design Manufacturing, Siloam Springs, AR) in groups of five or six with *ad libitum* access to tap water and irradiated lab chick diet (Harlan Teklad, Madison, WI). Brooder temperature was maintained at 32˚C to 35°C. Eight-week-old female CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Animals were housed in standard polycarbonate microisolator cages (Alternative Design Manufacturing, Siloam Springs, AR) containing aspen shredded bedding (Harlan Teklad, Madison, WI) in groups of three to four. Mice were fed a 4% standard rodent chow (Harlan Teklad, Madison, WI) with free access to food and water.

2.2 BACTERIAL STRAINS AND GROWTH CONDITIONS

The strains used in this study are summarized in Table 1. A spontaneously nalidixic acid resistant (Nal^r) strain of *Salmonella enterica* serovar Typhimurium ATCC14028s, HA420, which is virulent and persistent in murine models, was used as our wild type. Strains bearing a mutation of the *phoN* gene (ATCC14028s Δ*phoN*::Kan, HA431 and ATCC14028s Δ*phoN*::Cm, HA530) were constructed using the lambda red

recombinase method.[9] *phoN*+ and *phoN*- strains can be distinguished by blue-white selection on 5-bromo-4-chloro-3-indolyl phosphate (XP) containing media. *phoN*- strains form white colonies while *phoN*+ strains appear blue. The use of multiple antibiotic cassettes in generation of the mutant strains served as an additional tool for identification of wild type versus mutant colonies on selective media.

Salmonellae were routinely cultivated in Luria-Bertani (LB) broth at 37°C or 42°C, on LB plates and on Brilliant Green plates supplemented with the appropriate antibiotics. Bacterial cultures to be used to infect chicks or mice were grown overnight to stationary phase in LB broth supplemented with the appropriate antibiotics at 37°C or 42°C with aeration. Antibiotics and other supplements were used at the following concentrations: 20 mg/L XP and 100 mg/L nalidixic acid (Nal) or Brilliant Green agar containing 100 mg/L Nal and either 20 mg/L chloramphenicol (Cm) or 50 mg/L kanamycin (Kan).

Table 1. *S.* Typhimurium Strains Used in This Study.

Strain	Genotype	Reference
HA420	ATCC14028s Nal^r, wild type	Bogomolnaya et al. (in press)
HA431	ATCC14028sΔ*phoN*::Kan, wild type	This study
HA530	ATCC14028sΔ*phoN*::Cm, wild type	This study
HA460	ATCC14028sΔ*invA*::Kan	This study

2.3 P22 TRANSDUCTION

Prior to their use in experiments involving chicks, all mutants were transferred to a clean genetic background by P22 transduction. Briefly, the mutant strains were grown overnight as described above. Lysates were prepared using serial dilutions of P22 (10^{-1} to 10^{-4}) and 100µl of the overnight culture. Wild type HA420 was grown overnight and was used as the recipient strain. Recipient strain and lysate were mixed and incubated with LB and 10mM EGTA to inhibit lysis. The mixture was plated on LB with EGTA and the appropriate antibiotic marker and incubated as previously described. Colonies were picked for streaking on EBU plates with antibiotic to check for loss of phage. Once purification was determined, mutants were stored in 30% glycerol at -80°C until use.

2.4 SINGLE INFECTION

Thirty-six 1-week-old SPF White Leghorn chicks in groups of 6 were inoculated orally with $6x10^8$ CFU of HA420 in 0.1ml LB broth. Twenty-four control chicks in

groups of 4 were inoculated orally with 0.1ml sterile LB broth. Control birds were housed separately from infected birds to minimize cross contamination.

Six birds from the infected group and 4 from the control group were euthanized by CO_2 asphyxiation on days 1, 3, 6, 9 and 15 post infection. Liver, spleen, sections of large and small intestine and one cecal arm (cecum plus contents) were collected. A portion of each organ collected was weighed and placed in 3ml sterile PBS for bacteriology. Following homogenization, samples for bacteriology were serially diluted and plated on LB agar containing XP and Nal for determination of colony forming units (CFU).

Twelve female CBA/J mice were inoculated orally with 1.5×10^{11} CFU of HA420 in 0.1 ml sterile LB broth and 12 control mice were inoculated orally with 0.1ml sterile LB broth. Liver, spleen, cecum, and sections of small and large intestine were collected on days 1, 3, 9 and 15 days post infection. Organ samples were processed as described for chicks to enumerate CFU.

2.5 COMPETITIVE INFECTION

Twenty-seven 1-week-old SPF White Leghorn chicks were inoculated orally with 2×10^8 CFU of a 1:1 mixture of wild type HA530 and mutant HA460 in a volume of 0.1ml in sterile PBS. The inoculum was serially diluted for determination of CFU and the exact input ratio. Fecal samples were collected by cloacal swab on days 1, 3, 6, 9, 12 and 15 post infection. Samples were weighed and serially diluted. Double plating was performed on Brilliant Green agar containing Nal and Kan and Brilliant Green agar containing Nal and Cm for determination of CFU of wild type versus mutant strain.

Groups of five or six chicks were euthanized on days 1, 3, 6, 9, and 15 post infection and the cecum was collected. One cecal arm (cecum plus contents) from each chick was placed in 3ml sterile PBS. Samples were weighed, homogenized, serially diluted and plated as described for fecal samples. In order to determine how many CFU were luminal the contents from the second cecal arm for each chick was emptied into 3ml sterile PBS and the wall was then split into two sections. In order to determine cell associated CFU versus intracellular CFU one section of the cecal wall was washed and placed in 3ml sterile PBS and the remaining piece was washed and incubated in 3ml sterile PBS containing 30µl gentamicin at 37°C for 90 minutes. *Salmonella* CFU were determined for cecal content, cell associated with the cecal wall and intracellular in the cecal wall as described above.

Twenty 8-week-old female CBA/J mice were inoculated orally with 1.4×10^9 CFU of a 1:1 mixture of wild type strain HA431 and mutant strain HA460 in 0.1ml sterile PBS. Three fecal pellets (approximately 100mg) were collected from each mouse on days 1, 3, 6, 9, 12 and 15 post infection. Samples were weighed, serially diluted and plated. Four mice were euthanized on days 1, 3, 6, 9 and 15 post infection and the cecum was collected. Half of the cecum (cecum plus contents) from each mouse was weighed and placed in 3ml sterile PBS for plating as described for the fecal samples. In order to determine luminal, cell associated and intracellular CFU the contents from the remaining half of the cecum was placed in 3 ml sterile PBS and the remaining cecal wall was split into two sections for processing as described previously. All murine samples

were plated on LB agar containing Nal and XP for determination of colony forming units of wild type versus mutant strain.

2.6 STATISTICAL ANALYSIS

A two tailed Student's *t* test for independent samples was used to compare the differences in liver and spleen weights between infected and control groups of chicks and mice. *P* values of <0.05 were considered statistically significant.

Data obtained from competitive infection experiments were calculated as a mean log ratio of wild type versus mutant normalized to the input ratio. Statistical significance was determined using a two tailed Student's *t* test and *P* values of <0.05 were considered statistically significant (SPSS software, SPSS, Inc., Chicago, IL).

3. RESULTS

3.1 SYSTEMIC COLONIZATION BY *S.* TYPHIMURIUM

Organ weight and colonization were evaluated after euthanasia of infected and control animals. Hepatic and splenic enlargement is commonly found in mice infected with *S*. Typhimurium. This is most evident beginning at 15-30 days post infection in *Salmonella*-resistant murine models that do not develop systemic disease (unpublished data). Therefore, we examined the livers and spleens of our *S*. Typhimurium control and infected chicks and mice for increased size and gross pathology. The weight of liver and spleen from control and infected animals was evaluated (Figure 1; Figure 2). While the liver and spleen weights of both control and infected groups of chicks increased over time due to growth, we found that the spleens and livers of infected chicks were comparable in weight to those of uninfected age-matched chicks. In contrast, the liver and spleen weights of infected mice were greater than those of control mice. On day 15 post infection, the liver and spleen weight of infected mice was significantly greater ($P < 0.05$) than that of control mice.

S. Typhimurium colonization of liver and spleen in chicks and *Salmonella*-resistant CBA/J mice was also evaluated and correlated with the results of the previous paragraph detailing enlargement of systemic organs in mice alone. In both chicks and mice, the systemic organs were poorly colonized at early time points post infection (Figure 3). This low level of colonization of systemic organs was maintained in chicks while the level of colonization of the liver and spleen in mice increased through day 15 post infection when these organs were also significantly enlarged.

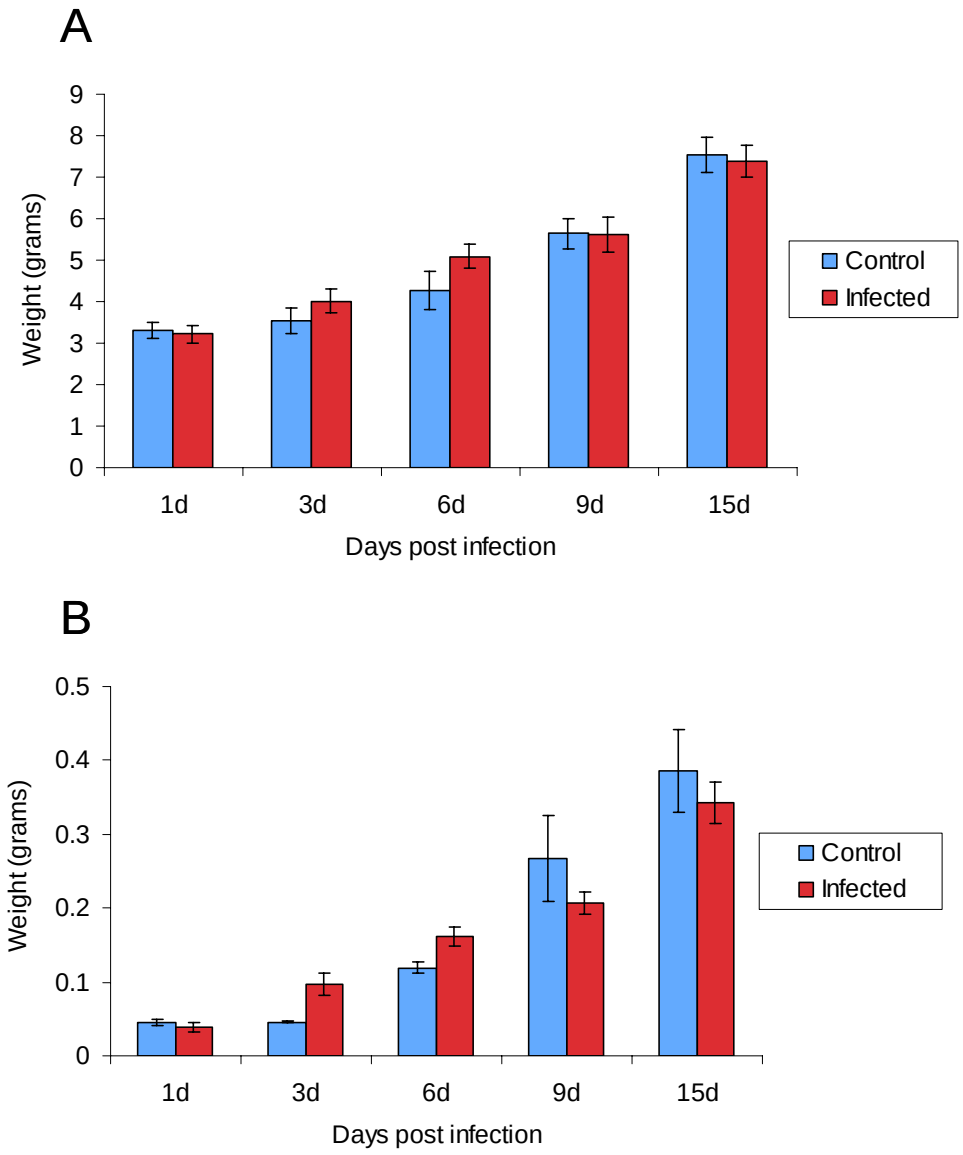

Figure 1. Organ Weights Following *S.* Typhimurium Infection in Chicks. Data are shown as mean liver (A) and spleen (B) weight in grams. Error bars denote standard error.

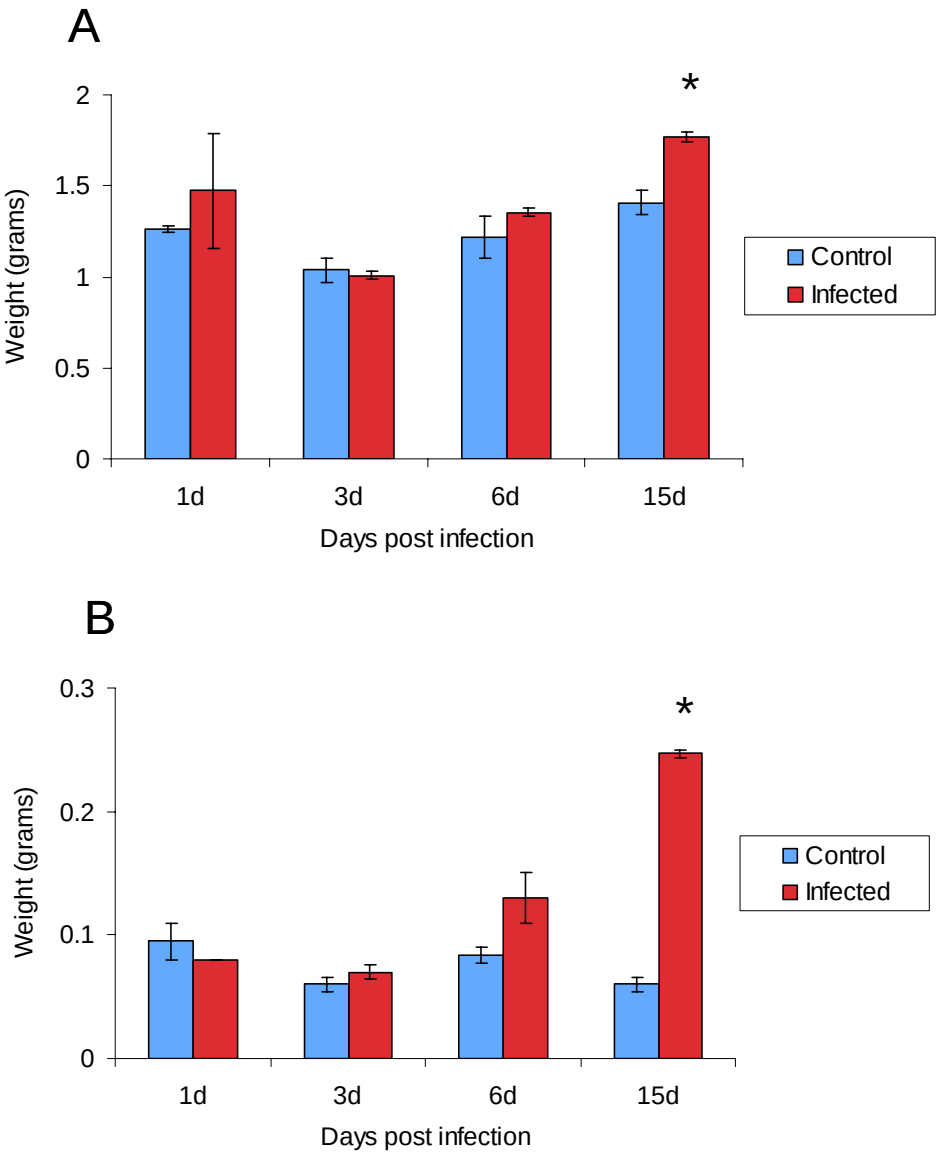

Figure 2. Organ Weights Following *S.* Typhimurium Infection in Mice. Data are shown as mean liver (A) and spleen (B) weight in grams. Error bars denote standard error and asterisks indicate a statistically significant difference between control and infected groups at $P < 0.05$.

3.2 DYNAMICS OF *S.* TYPHIMURIUM INFECTION

We analyzed organ colonization of *S.* Typhimurium in liver, spleen, small intestine (SI), cecum and large intestine (LI) of infected chicks and *Salmonella*-resistant CBA/J mice (Figure 3). The ceca were the most heavily colonized organ at all time points post infection in both models of infection. In 1-week-old chicks, the ceca had a bacterial burden which was heaviest until day 3 post infection but appeared to be reduced from this point until the end of the experiment on day 15 post infection (Figure 3A). In *Salmonella*-resistant mice however, the number of *Salmonella* in the cecum steadily increased throughout the duration of the experiment (Figure 3B). The colonization of the small intestine and large intestine in the chick followed similar dynamics to cecal colonization in this model in that the number of organisms in these niches initially increased until day 3 post infection and then decreased over the remainder of the experiment (Figure 3A). In *Salmonella*-resistant CBA/J mice, colonization of the small and large intestine increased throughout the duration of the experiment (Figure 3B) in contrast to the chick.

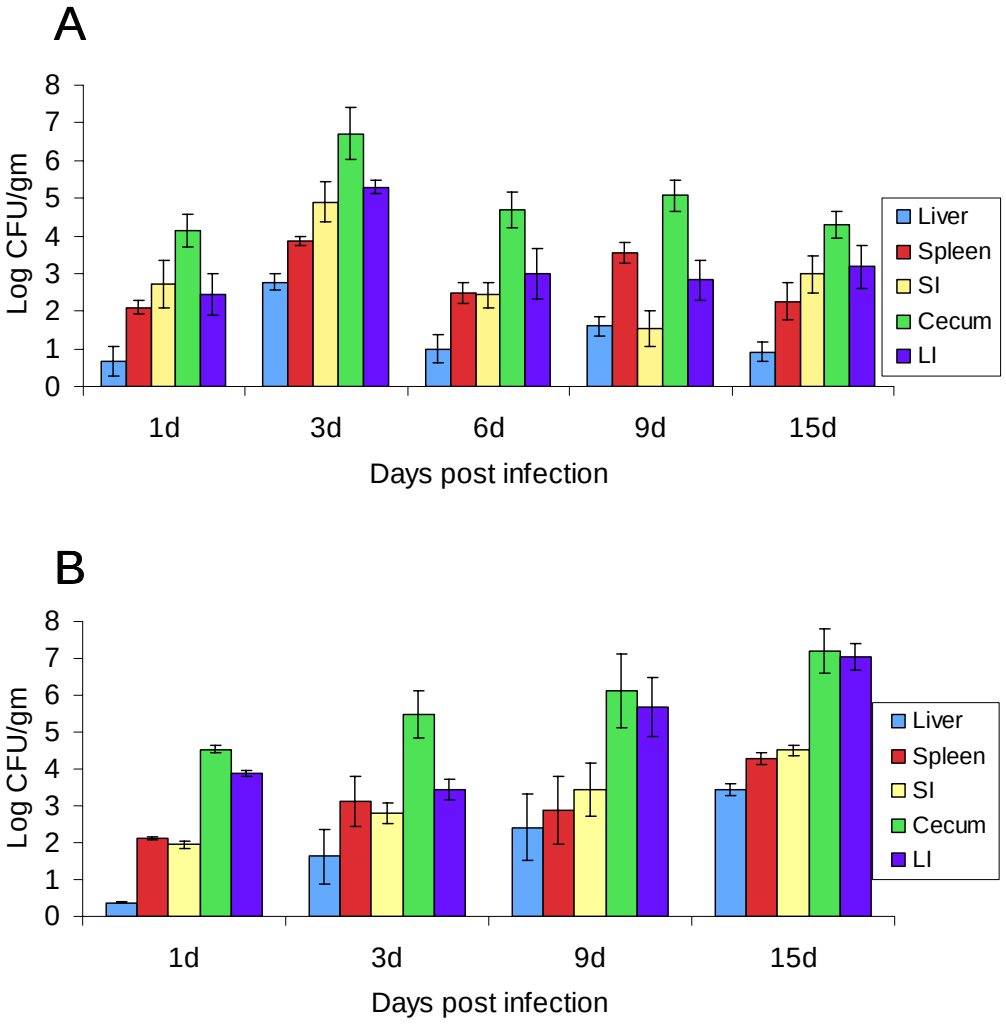

Figure 3. Organ Colonization Dynamics of *S.* Typhimurium in Chicks and Mice. Recovery of *S.* Typhimurium following oral inoculation in chicks (A) and mice (B) is shown as mean CFU/gm of organ tissue. Error bars denote standard error.

3.3 THE INFLUENCE OF SPI-1 IN CECAL COLONIZATION

In order to determine the importance of utilization of the intracellular niche by *S.* Typhimurium in cecal colonization we studied the distribution of wild type *S.* Typhimurium versus a mutant in *invA*. Δ*invA* mutants produce a defective type III secretion system and although they adhere well to cultured epithelial cells, they are unable to invade these cells.[13,15] During a competitive infection using wild type *S.* Typhimurium ATCC14028s versus an isogenic Δ*invA* mutant we analyzed the distribution of both strains in the whole cecum, cecal contents and cecal epithelium (both cell associated and intracellular fractions). The number of wild type and mutant CFU recovered from each component, as well as the ratio of wild type to mutant in each sample, was determined.

3.3.1 *Colonization of Wild Type during Competitive Infection*

First, we compared cecal colonization by total numbers of wild type *S.* Typhimurium in chicks and mice (Figure 4A; Figure 5A). On day 1 post infection the ceca of both animal models we used were heavily colonized with approximately 10^6 CFU/gm and 10^5 CFU/gm primarily in the cecal contents. As we noted previously, the colonization dynamics in the cecum differed between the two host species we studied.

In chicks, cecal CFU of *S.* Typhimurium remained high to day 6 post infection and then decreased for the duration of the experiment ending at 15 days post infection (Figure 4A, blue and red bars). At day 15 post infection, cecal CFU of *S.* Typhimurium in chicks had fallen to very low levels and was nearly undetectable. The largest proportion of bacteria was consistently located within the cecal contents (Figure 4A, red

bars). The number of cell associated but extracellular *Salmonella* in the chick intestine was unexpectedly high until 9 days post infection (Figure 4A, yellow bars). Of the minority of bacteria associated with the cecal epithelium, most were extracellular, thus not protected from gentamicin killing (Figure 4A, yellow and green bars) while near the termination of the experiment nearly all of the *Salmonella* remaining associated with the epithelium were intracellular (Figure 4A, green bars).

The colonization of the murine cecum by *S.* Typhimurium had different dynamics (Figure 5A, blue and red bars). In mice, cecal CFU of *S.* Typhimurium was also primarily in the cecal contents, however bacterial CFU increased throughout the duration of the experiment to day 15 post infection. In infected mice, the cell associated and intracellular bacterial counts were a very small proportion of the total *S.* Typhimurium CFU (Figure 5A, yellow and green bars).

3.3.2 *Colonization of* ΔinvA *during Competitive Infection*

We also studied the contribution of the SPI-1 encoded type III secretion system in colonization of the cecum in both chicks and *Salmonella*-resistant mice. We examined whole cecum, cecal contents, epithelium associated and intracellular colonization during competitive infection between wild type *S.* Typhimurium ATCC14028 and an otherwise isogenic Δ*invA* mutant. Initial colonization by the Δ*invA* mutant in the cecum (whole cecum and its contents) was approximately equal to that of wild type *S.* Typhimurium at day 1 post infection in both chicks and mice (Figure 4, blue bars; Figure 5, blue bars). In chicks, colonization of wild type and Δ*invA* mutant remained equal until day 9 post infection when Δ*invA* mutant levels fell dramatically. In

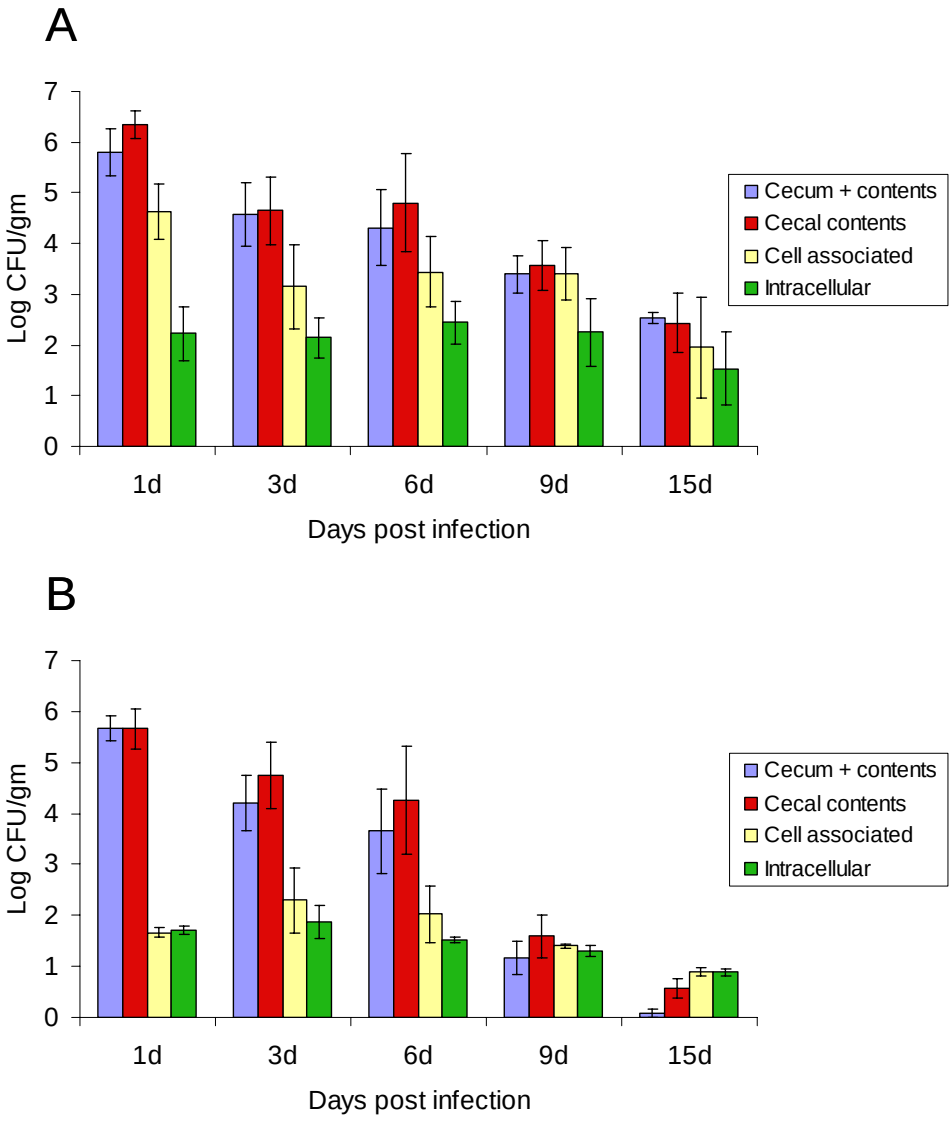

Figure 4. Differential Cecal Colonization of *S.* Typhimurium in Chicks. Recovery of wild type (A) and Δ*invA* mutant (B) from the cecum in chicks following oral inoculation is shown as mean CFU/gm of cecal tissue or cecal contents. Error bars denote standard error.

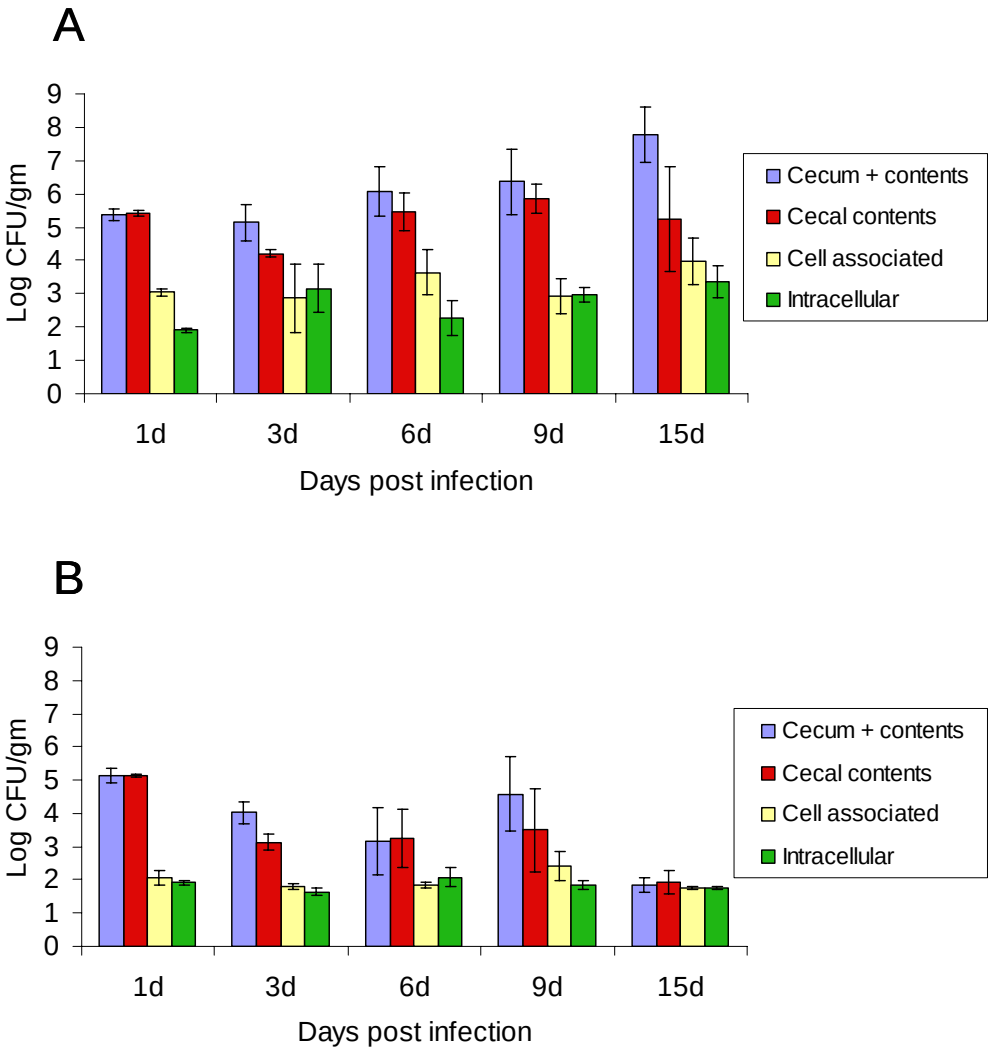

Figure 5. Differential Cecal Colonization of *S.* Typhimurium in Mice. Recovery of wild type (A) and Δ*invA* mutant (B) from the cecum in mice following oral inoculation is shown as mean CFU/gm of cecal tissue or cecal contents. Error bars denote standard error.

mice however, Δ*invA* mutant levels declined more gradually throughout the experiment. The Δ*invA* mutant exhibited a more dramatic defect in our murine model (Figure 5B) than in our chick model (Figure 4B) at 15 days post infection. This may be attributable to the greater importance of intracellular replication in the epithelium of the murine cecum versus the chick cecum. However, we note that the levels of luminal bacteria in the chick at this time point were low, reducing the dynamic range of this assay in the chick model.

Over the first 6 days of infection the number of Δ*invA* mutants in the cecal contents in chicks remained comparable to the level of the isogenic wild type in that compartment over the same duration and then fell dramatically to lower levels than the number of wild type organisms during the completion of the experiment (Figure 4A, B, red bars). In mice, although wild type and Δ*invA* mutants colonized equally well on day 1 post infection, the number of wild type *S.* Typhimurium in the cecal contents rose dramatically throughout the duration of the experiment (Figure 5A, red bars). This was not the case for the Δ*invA* mutant however as the numbers of mutant in the cecal contents remained essentially the same through day 9 post infection (Figure 5B, red bars).

Despite the presence of high numbers of Δ*invA* mutants in the lumen of the cecum at day1 post infection in chicks, the number of cell associated bacteria was markedly decreased as compared to wild type (Figure 4A, B, yellow and green bars). In mice at day 1 post infection, the CFU of Δ*invA* mutant associated with the cecal epithelium was also significantly lower than the number of cell associated wild type

organisms (Figure 5A, B, yellow and green bars). In both chicks and mice, the number of Δ*invA* mutants associated with the cecal epithelium remained at low levels throughout the duration of the experiment. However, the number of both wild type and Δ*invA* mutants associated with the cecal wall in the chick was very low throughout this experiment, thus it is difficult to definitively assign the importance of having a functional SPI-1 type III secretion system for colonization of the cecal epithelium in the chick.

3.3.3 *Competitive Index of Wild type versus* ΔinvA

For ease of interpretation, we also expressed the data from our competitive infection between wild type *S.* Typhimurium ATCC14028 and our Δ*invA* mutant as the normalized log (wild type CFU/ Δ*invA* mutant CFU) for each cecal compartment we analyzed in these experiments. We have noted previously that in both mice and chicks the Δ*invA* mutant initially colonizes the whole cecum in numbers equal to the wild type. However, the Δ*invA* mutant does exhibit a colonization defect in the cecum in both chicks and mice over time with a significant difference ($P < 0.05$) on days 9 and 15 in chicks and on day 15 post infection in mice (Figure 6A, B). The colonization defect manifested by the Δ*invA* mutant in the murine cecum appears to be much larger then the defect manifested in the chick ceca (5 logs versus 2 logs). However, the magnitude of this problem may appear to be lower in chicks because their ceca were not as well colonized at this time point leading to lower dynamic range in the assay.

Analysis of the cecal contents only showed a similar pattern of colonization of wild type *S.* Typhimurium and Δ*invA* mutant to that seen in the whole cecum in both

host species. In chicks, there was no significant difference between wild type and mutant except on day 9 post infection and this was a 2 log defect (Figure 7A). In mice, wild type *S.* Typhimurium was over represented compared to Δ*invA* mutant at all time points in the cecal contents, with significant differences ($P < 0.05$) on days 1, 3, 6 and 9 post infection (Figure 7B).

Analysis of the cell associated component of *S.* Typhimurium and Δ*invA* mutant growth in the cecum included bacteria that were firmly attached to the epithelial cell wall and those that were intracellular (Figure 8). In both chicks and mice there was a significant difference ($P < 0.05$) between wild type and mutant CFU in the cell associated compartment on day 1 post infection. Wild type had a 1000 fold advantage over Δ*invA* mutant in chicks at this early time point following infection. There was also a significant difference ($P < 0.05$) in cell associated numbers between the two strains on days 6 and 9 in chicks (Figure 8A) and in mice on day 15 post infection (Figure 8B).

Intracellular bacterial counts of *S.* Typhimurium versus Δ*invA* mutant were analyzed following a gentamicin protection assay to kill all extracellular bacteria associated with the cecal cell wall. The difference between wild type and mutant was not statistically significant for chicks (Figure 9A). In mice, there was a statistically significant difference ($P < 0.05$) on days 9 and 15 post infection (Figure 9B). The intracellular bacterial counts were very low at all time points for both chicks and mice.

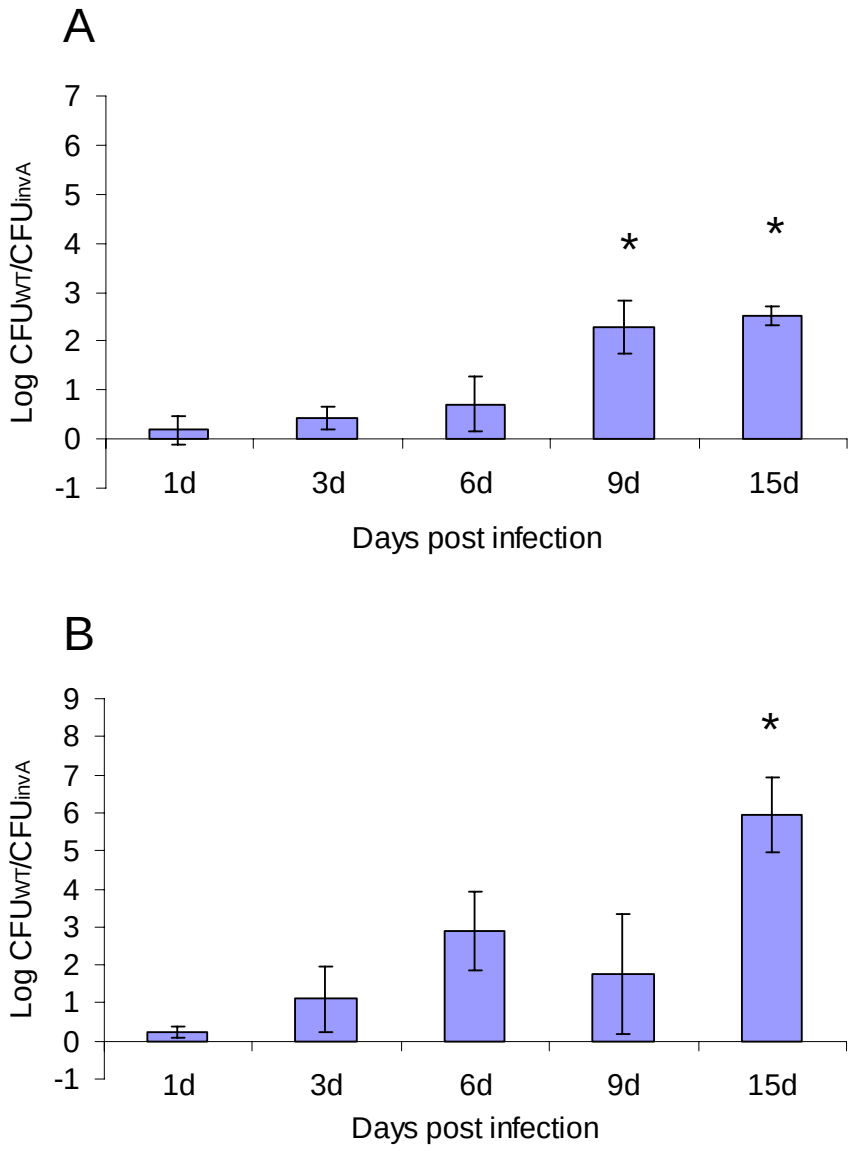

Figure 6. Competitive Cecal Colonization. Recovery of *S.* Typhimurium and Δ*invA* mutant in the whole cecum in chicks (A) and mice (B) is shown as the mean log ratio of wild type versus mutant. Error bars denote standard error and asterisks indicate statistical significance between the ratio of wild type versus Δ*invA* mutant in the cecum at each time point post infection at $P < 0.05$.

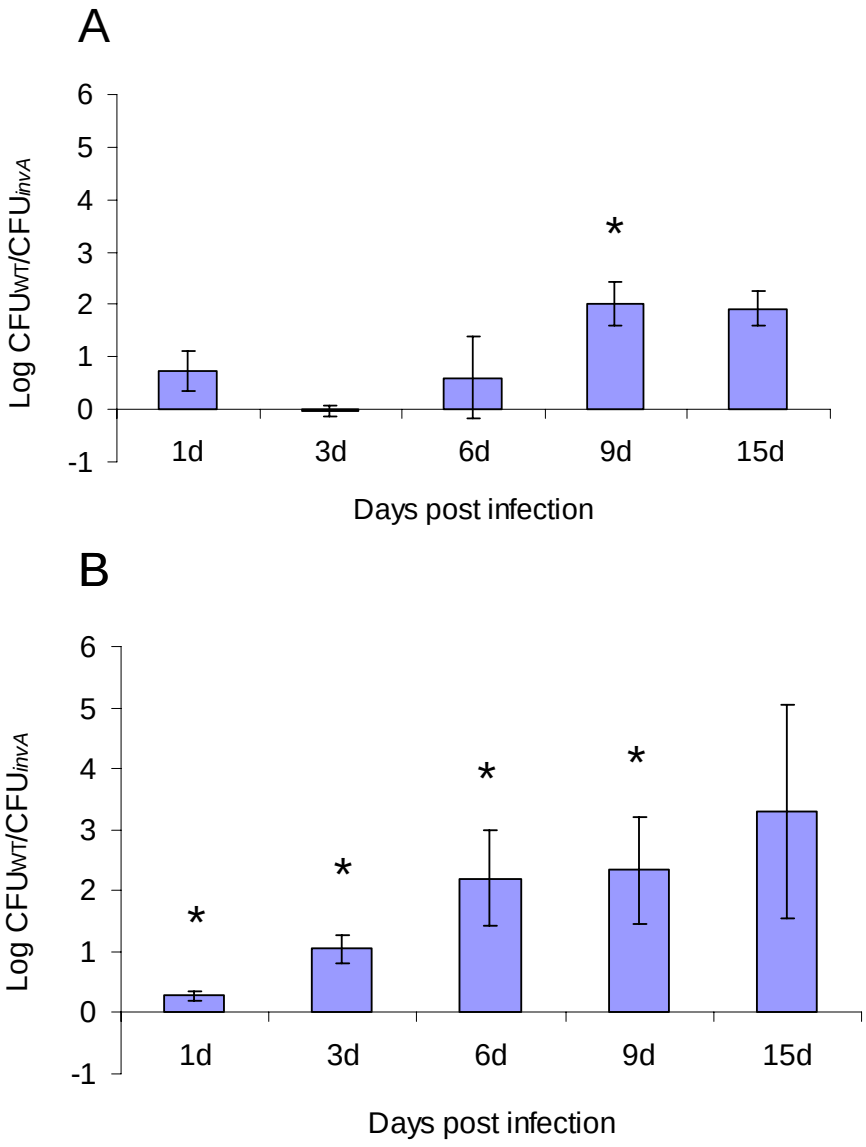

Figure 7. Competitive Growth in Cecal Contents. Recovery of *S.* Typhimurium and Δ*invA* mutant in the cecal contents in chicks (A) and mice (B) is shown as the mean log ratio of wild type versus mutant. Error bars denote standard error and asterisks indicate statistical significance between the ratio of wild type versus Δ*invA* mutant in the inoculum and the corresponding ratio in the cecal contents at each time point post infection at $P < 0.05$.

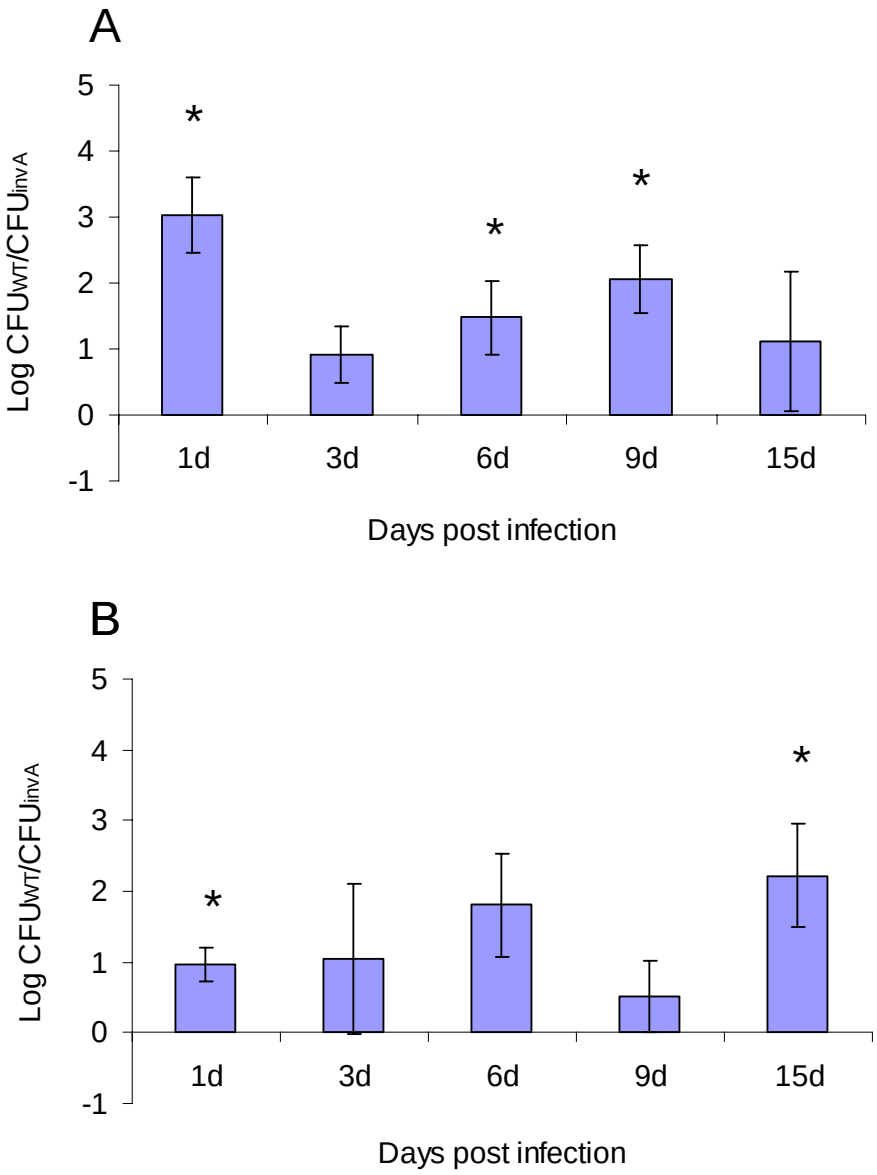

Figure 8. Competitive Cell Associated Growth. Recovery of *S.* Typhimurium and Δ*invA* mutant associated with the cell wall in chicks (A) and mice (B) is shown as the mean log ratio of wild type versus mutant. Error bars denote standard error and asterisks indicate statistical significance between the ratio of wild type and Δ*invA* mutant in the inoculum and the corresponding ration in the cell associated component of the cecum at each time point post infection at $P < 0.05$.

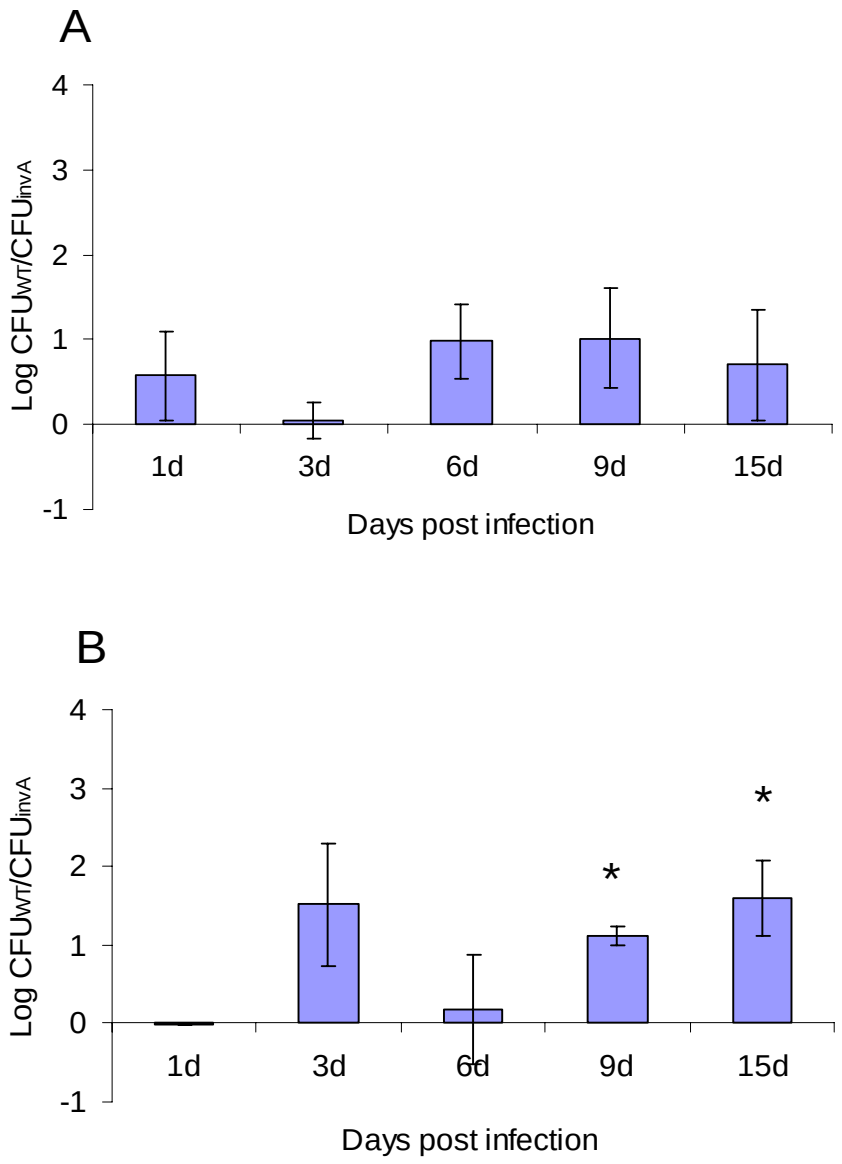

Figure 9. Competitive Intracellular Growth. Recovery of intracellular *S.* Typhimurium and Δ*invA* mutant in chicks (A) and mice (B) is shown as the mean log ratio of wild type versus mutant. Error bars indicate standard error and asterisks indicate statistical significance between the ratio of wild type versus Δ*invA* mutant in the inoculum and the corresponding ration in the intracellular component of the cecum at each time point post infection at $P < 0.05$.

3.3.4 *Fecal Shedding of Wild Type versus* ΔinvA

We analyzed the level of fecal shedding of wild type *S*. Typhimurium ATCC14028 and our Δ*invA* mutant. Bacterial counts of wild type and Δ*invA* mutant recovered from each animal were analyzed individually and as the ratio of wild type to mutant in each sample. In general, the overall level of fecal shedding of wild type *S.* Typhimurium was greater in mice than in chicks (Figure 10; Figure11). In addition, in mice the level of fecal shedding correlated more accurately with the level of cecal colonization than the equivalent measures in chicks. On day 1 post infection the feces collected from chicks contained approximately 10^3 CFU/gm and that of the mice over 10^5 CFU/gm. While the mean bacterial counts in chicks remained relatively unchanged through day 12 post infection, they increased in mice to approximately 10^7 CFU/gm of feces on day 6 post infection where they remained through day 15. On day 15, there was no *S.* Typhimurium detected in the feces of chicks.

Fecal shedding of Δ*invA* mutant in chicks and mice is shown in Figures 10 and 11. On day 1 post infection, bacterial counts of Δ*invA* mutant were at approximately the same level as was wild type for both chicks and mice at 10^3 and 10^5 CFU/gm of feces, respectively. In addition, Δ*invA* mutant was shed in feces to similar levels as wild type throughout the duration of the experiment in the chicks. This was not the case in mice, where Δ*invA* mutant was shed at lower levels than the isogenic wild type beginning on day 6 post infection. On day 15 post infection chicks were not shedding detectable levels of wild type or Δ*invA* mutant.

We show this analysis of *S.* Typhimurium versus Δ*invA* mutant shed in the feces of chicks and mice plotted as a normalized log (CFU WT/CFU Δ*invA*). In chicks, there was no difference in the levels of wild type *S.* Typhimurium and mutant shed in the feces on day 1 post infection as we have previously noted (Figure 12A). Although bacterial counts were low, wild type had a very mild but statistically significant ($P < 0.05$) advantage over Δ*invA* mutant in the feces of chicks on day 3 post infection, but not at any other time points. Chicks were not shedding *S.* Typhimurium or Δ*invA* mutant on day 15 post infection. In mice, wild type *S.* Typhimurium had an advantage over Δ*invA* mutant at all time points with a statistically significant ($P < 0.05$) difference on days 1, 3, 6 and 9 post infection (Figure 12B).

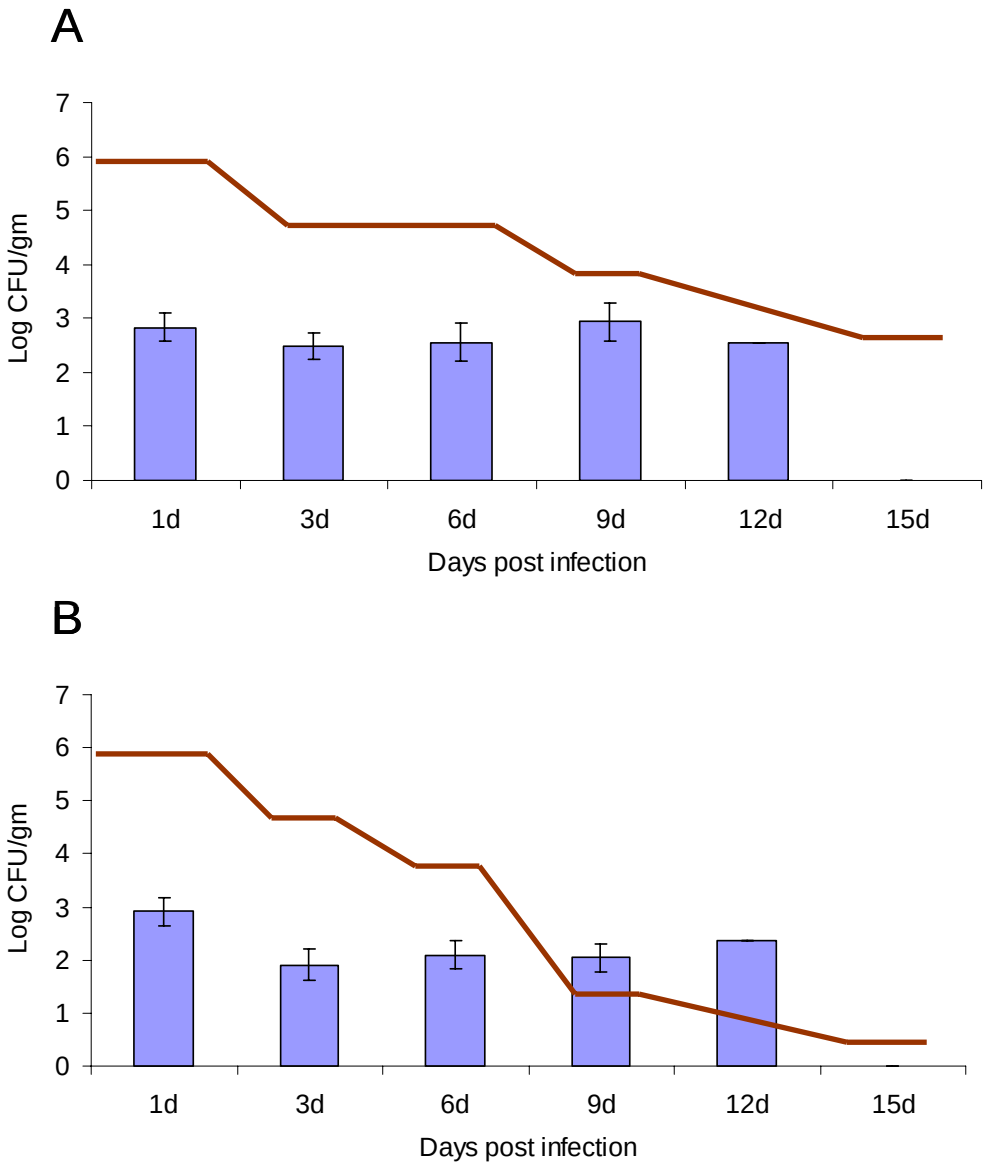

Figure 10. Dynamics of *S.* Typhimurium Fecal Shedding in Chicks. Recovery of wild type (A) and Δ*invA* mutant (B) from feces in chicks following oral inoculation is shown as mean CFU/gm of feces. Error bars denote standard error and lines indicates the corresponding level of cecal colonization.

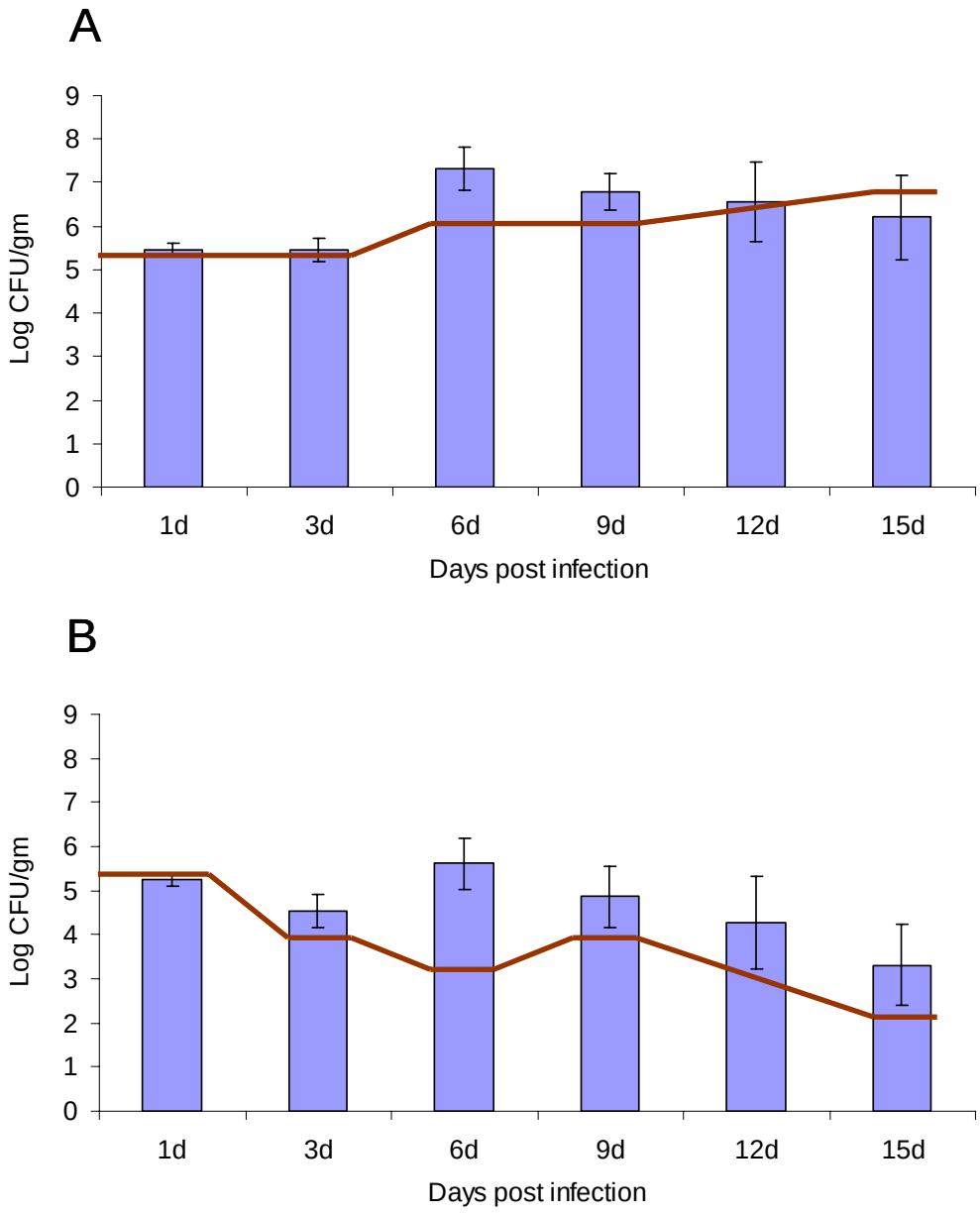

Figure 11. Dynamics of *S.* Typhimurium Fecal Shedding in Mice. Recovery of wild type (A) and Δ*invA* mutant (B) from feces in mice following oral inoculation is shown as mean CFU/gm of feces. Error bars denote standard error and lines indicates the corresponding level of cecal colonization.

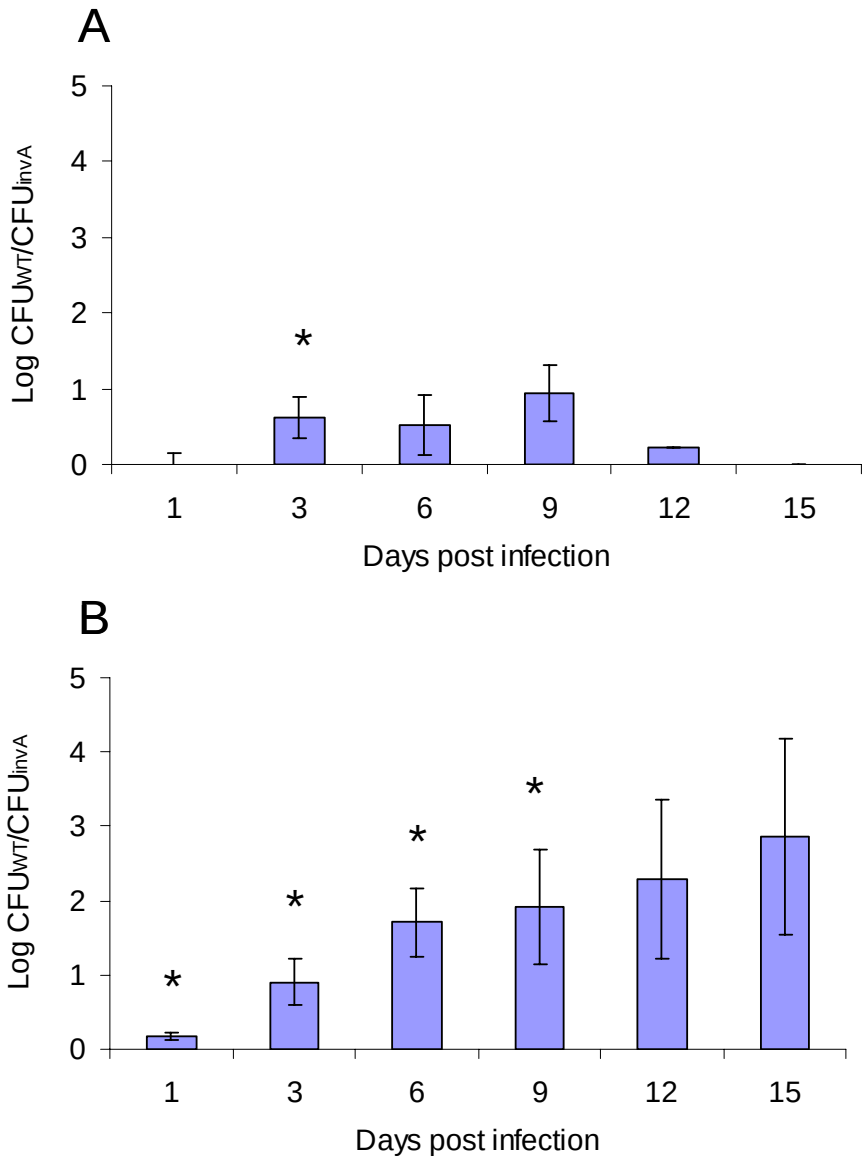

Figure 12. Competitive Fecal Shedding. Recovery of *S.* Typhimurium and Δ*invA* mutant in the feces of chicks (A) and mice (B) is shown as the mean log ratio of wild type versus mutant. Error bars denote standard error and asterisks indicate statistical significance between the ratio of wild type versus Δ*invA* mutant in the inoculum and the corresponding ratio in the feces at each time point post infection at $P < 0.05$.

4. DISCUSSION AND CONCLUSIONS

The goal of this study was to develop a 1-week-old SPF White Leghorn chick model of *S.* Typhimurium ATCC14028 infection. We used this model to investigate the location of *S.* Typhimurium following oral inoculation in White Leghorn chicks and compared these findings to the location of *S.* Typhimurium in *Salmonella*-resistant CBA/J mice. We also compared the distribution of SPI-1 mutants in the cecum in chicks and mice and evaluated levels of fecal shedding of our wild type *S.* Typhimurium and SPI-1 mutant strains.

Our results indicate that in chicks, *S.* Typhimurium ATCC14028 is found in the greatest numbers in the cecum rather than in other portion of the intestinal tract or in systemic organs. Within the cecum of chicks, bacteria were largely located in the cecal contents with a smaller number associated with the cell wall. Our data show that intracellular localization is not a prominent niche for colonization of the chick cecum by *S.* Typhimurium ATCC14028. Our results using ATCC14028, one of the most commonly studied *S.* Typhimurium isolated in laboratory animals and *in vitro*, confirm earlier studies that found the ceca to be the major site for recovery of various Typhimurium isolates in chicks.[3,10,34] Furthermore, sampling the cecal contents has been shown to be the best method for recovering *Salmonella* in the gastrointestinal tract in chicks[7,11] and we show that evaluating fecal shedding of *S.* Typhimurium in chicks does not accurately reflect colonization of the cecum by this organism.

Greater numbers of *S.* Typhimurium were also found in the cecal contents of *Salmonella*-resistant CBA/J mice with very few CFU associated with the cecal wall,

either attached to the wall or intracellular, except on day 3 post infection when there was an increase in the number of cell associated bacteria. This time point may be important for bacterial spread from the gastrointestinal tract to systemic organs in mice. We have also shown that in CBA/J mice infected with *S.* Typhimurium ATCC14028 organisms were located in the liver and spleen by day 15 post infection.

We also investigated the importance of SPI-1, which encodes the TTSS-1 type III secretion system, in an *S.* Typhimurium infection in chicks and mice. The SPI-1 TTSS is important for colonization of mammalian cells,[13,14,15,37,38] but the role of this important virulence factor in chickens is less clear. Factors such as age and genetic background of the chick as well as the *S.* Typhimurium isolate used for infection may influence the relative importance of SPI-1.

The results of our study indicate that SPI-1 is important for cecal persistence in *Salmonella*-resistant CBA/J mice. *S.* Typhimurium had a statistically significant advantage over Δ*invA* mutants in the whole cecum and cecal contents in this model. *S.* Typhimurium ATCC14028 also had a small advantage over Δ*invA* mutants in the cell associated component of the cecum in mice.

In contrast to our findings in murine models, our data indicate that having an intact TTSS-1 may not be as critical for total intestinal colonization in chicks as it is in mice. While SPI-1 was not necessary for successful infection in 1-week-old White Leghorn chicks, our data show that SPI-1 is important for bacterial attachment to the cell wall in this model. This finding in chicks conflicts with previous studies utilizing cultured mammalian epithelial cells which indicated that while Δ*invA* mutants were

deficient in their ability to invade eukaryotic cells, they were not defective in their ability to attach to cultured eukaryotic cells.[13,15] From our experiments it appears that wild type *S.* Typhimurium ATCC14028 has a modest but significant advantage over a SPI-1 mutant in chicks in the whole cecum and cecal contents at the later time points of infection. However, our results should be interpreted in light of the low level of colonization of our chicks by both wild type *S.* Typhimurium and SPI-1 mutant in our assay.

We studied the congruence between cecal colonization and fecal shedding of *S.* Typhimurium ATCC14028 in both chicks and mice. While the bacterial numbers of *S.* Typhimurium and Δ*invA* mutant in the feces of mice reflected those in the whole cecum, we show that this is not true for chicks. The bacterial counts in the feces of chicks were consistently two orders of magnitude less than those seen in the cecum. Since sampling the feces is a common method used to determine extent of infection during *S.* Typhimurium persistence studies, this has important practical implications for performing these studies in chicks. Sampling the feces rather than the cecum is unlikely to give an accurate representation of the extent of colonization in this species.

In conclusion, the cecum, specifically the cecal contents, is the major organ for recovery of *S.* Typhimurium in 1-week-old White Leghorn chicks following oral inoculation. Additionally, the cecal contents are the major site for recovery of *S.* Typhimurium in the cecum of *Salmonella*-resistant mice. We also confirm that SPI-1 is important for successful infection in mice and, although SPI-1 is not necessary for successful infection in chicks overall, it may play a role in bacterial attachment to the

cecal cell wall during infection. Further work is needed to determine genes that are important for intestinal colonization and persistence in the chick.

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